AD_____

CONTRACT NUMBER: DAMD17-95-C-5004

TITLE: Neuropharmacological Characterization of Botulinum Neurotoxin

PRINCIPAL INVESTIGATOR: Lance L. Simpson, Ph.D.

CONTRACTING ORGANIZATION: Thomas Johnson University
Jefferson Medical College
Philadelphia, Pennsylvania 19107-6799

REPORT DATE: April 1996

TYPE OF REPORT: Midterm

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 1996	Midterm (1 Apr 95 - 31 Mar 96)

4. TITLE AND SUBTITLE
Neuropharmacological Characterization of Botulinum Neurotoxin

5. FUNDING NUMBERS
DAMD17-95-C-5004

6. AUTHOR(S)
Lance L. Simpson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Thomas Jefferson University
Jefferson Medical College
Philadelphia, Pennsylvania 19107-6799

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Experiments have been done to clarify the nature of botulinum toxin binding. This work is an essential prelude to efforts to identify drugs that can antagonize toxin action by blocking toxin association with cell surface receptors. During the first part of the work, experiments were done: *i)* to identify human tissue preparations that can be used to study electrophysiology, ligand binding, and certain aspects of molecular biology, and *ii)* to examine botulinum toxin action on these tissues. This work resulted in the findings that the human pyramidalis muscle can be used as a model to study neuromuscular transmission, and cerebellar synaptosomes can be used as models to study CNS function. During the second part of the work, the process of characterizing toxin receptors began. This work resulted in the finding that: *i)* toxin receptors are found in most parts of the nervous system, but distribution is very uneven, and *ii.)* molecular biology techniques can be used to transfer functioning receptors from the nervous system to a model translation system, i.e., Xenopus oocytes.

14. SUBJECT TERMS
Botulinum toxin
Neuromuscular junction
Receptor

15. NUMBER OF PAGES
54

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Lance Simpson 4/19/96

PI - Signature Date

TABLE OF CONTENTS

GENERAL INTRODUCTION

The overall goal of the research is to identify clinically relevant approaches to preventing or treating the poisoning due to botulinum toxin. During the first half of the contract, the work was divided into two phases, as follows:

1) identification of human tissues that are susceptible to botulinum toxin poisoning, and

2) use of human tissues and laboratory animal tissues in the effort to characterize toxin binding sites

The two major components of the work are described in the two sections of this report.

SECTION 1

CHARACTERIZATION OF BOTULINUM TOXIN ACTION ON HUMAN TISSUES

ABSTRACT

In the present study, electrophysiologic techniques were used to monitor toxin effects on endplate activity in surgically excised human pyramidalis muscles, ligand binding studies were done to detect and characterize toxin receptors in human nerve membrane preparations, and molecular biological techniques were used to isolate and sequence a human gene that encodes a substrate for botulinum neurotoxin. The results demonstrated that stable resting membrane potentials (-61.5 mV; SEM $\pm$ 0.7) were maintained in individual fibers of pyramidalis muscle for up to 6 hours at 33°C. The rate of spontaneous miniature endplate potentials was low in physiological solution (0.14 per second) but increased in response to elevations in extracellular potassium concentration. In keeping with epidemiologic findings, botulinum toxin type A (10^{-8} M) paralyzed transmission in human preparations (ca. 90 min). In contrast to epidemiologic findings, serotype C (10^{-8} M) also paralyzed human tissues (ca. 65 min). Iodinated botulinum neurotoxin types A and C displayed high affinity binding to receptors in human nerve membrane preparations. In addition, the human nervous system was found to encode polypeptides that are substrates for botulinum neurotoxin types A (SNAP-25) and C (syntaxin 1A). These data have important implications bearing on: *1)* the development and administration of vaccines against botulism, and *2)* the testing of toxin serotypes for the treatment of dystonia.

INTRODUCTION

Botulinum neurotoxin exists in seven different serotypes, designated A, B, C, D, E, F and G. All seven serotypes are large proteins that act on cholinergic neuromuscular junctions to block transmitter release. Research on laboratory animal preparations has shown that the toxins produce this effect by proceeding through a sequence of four steps, as follows: *1)* binding to receptors on the plasma membrane, *2)* penetration of the plasma membrane by receptor-mediated endocytosis, *3)* penetration of the endosome membrane by pH-induced translocation, and *4)* intracellular expression of an enzymatic action that culminates in blockade of exocytosis (Simpson, 1980; Simpson, 1981; Habermann and Dreyer, 1986; Simpson, 1989).

A great deal of attention has recently been focused on botulinum neurotoxin. This is due in part to the discovery that the various serotypes are zinc-dependent endoproteases that cleave synaptic proteins implicated in docking and fusion of vesicles (Schiavo *et al.*, 1994b). Serotypes A and E cleave SNAP-25 (Blasi *et al.*, 1993a; Schiavo *et al.*, 1993a; Binz *et al.*, 1994); serotype C acts on syntaxin (Blasi *et al.*, 1993b); and serotypes B, D, F and G act on synaptobrevin (Schiavo *et al.*, 1993a; Schiavo *et al.*, 1992; Yamasaki *et al.*, 1994a; Schiavo *et al.*, 1993b; Yamasaki *et al.*, 1994b; Schiavo *et al.*, 1994a). SNAP-25, syntaxin, and synaptobrevin, along with several other polypeptides, are thought to be essential for exocytosis (Söllner *et al.*, 1993b; Südhof *et al.*, 1993; Söllner *et al.*, 1993a; Pevsner *et al.*, 1994).

Another reason for the recent focus on botulinum neurotoxin is its introduction as a therapeutic agent for the treatment of dystonia (Jankovic and Brin, 1991; Jankovic and Hallett, 1994). Medically supervised administration of toxin is used to produce local blockade of transmission at sites of excessive efferent activity. Botulinum neurotoxin is now regarded as the treatment of choice for disorders such as blepharospasm, strabismus, and hemifacial spasm, and it is likely to be accepted as the treatment of choice for many other neurological disorders (American Academy of Neurology, 1990; NIH Consensus Statement, 1991).

In spite of the fact that botulinum neurotoxin is both an agent that causes disease and a drug that has been approved for medicinal use, its actions have never been systematically studied on viable human tissues, such as the neuromuscular junction. As a result, there is a profound lack of knowledge about many of the most fundamental properties of the toxin. For example, there exist no data on the comparative dose-response characteristics of the seven toxin serotypes. Indeed, there are no convincing data to demonstrate whether the human neuromuscular junction is actually sensitive to all seven serotypes. As a consequence, epidemiologic findings on the occurrence of food-borne botulism have been used as the basis for deciding which serotypes should be tested as therapeutic agents. To date, epidemiologic data suggest that serotypes A, B, E, F and G cause adult botulism, whereas serotypes C and D do not (Gangarosa *et al.*, 1971; Dowell, Jr. 1984).

The goal of the present study was to undertake the first systematic analysis of botulinum toxin action on isolated and viable human neuromuscular junctions. Serotype A, which has often been implicated in naturally-occurring botulism, and serotypes C and D, which have rarely if ever been implicated, were selected for evaluation. Because this is the first study to undertake a detailed examination of toxin action on living human tissues, the work was divided into two phases: *1)* identification and characterization of a human preparation that is suitable for analyzing neuromuscular transmission, and *2)* examination of botulinum toxin types A, C, and D action on this preparation.

MATERIALS AND METHODS

Human tissues. Institutional Review Board approval was obtained for protocols in which striate muscle was removed during surgical procedures (e.g., removal of pyramidalis muscle from patients undergoing laparotomies). Informed consent was obtained whenever removal of tissue was not an essential part of the surgical procedure.

Excised tissues were immersed in chilled physiological solution of the following composition (mM): NaCl, 138.8; KCl, 4.0; KH_2PO_4, 1.0; $NaHCO_3$, 12.0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; and glucose, 11.0. Depending on size, each preparation was divided into smaller fascicles approximately 5 mm wide and 1 mm thick. Individual fascicles were pinned in a 35 mm Sylgard-coated Petri dish and continuously perfused (3 ml per min) with fresh physiological solution bubbled with 95% O_2 and 5% CO_2. The bath temperature was maintained at 33°C unless otherwise stated.

Electrophysiology. Standard electrophysiological techniques were used to record endplate activity. Glass microelectrodes filled with 3 M KCl (tip resistance 20 - 40 MΩ) were connected to a high input impedance amplifier. The output from the amplifier was further amplified, filtered through a low pass filter and digitized through an A/D converter interfaced with a computer. Data acquisition, storage and analysis were achieved with AXOTAPE and PCLAMP software (Axon Instruments, Inc.).

Endplate regions were localized by following the course of tiny intramuscular nerve branches along the surface of muscle fibers. Microelectrodes were inserted as close as possible to endplate regions, and the rate of spontaneous miniature endplate potentials (MEPPs) was monitored at various levels of extracellular potassium (see Results). During a typical experiment, MEPPs were measured for a baseline period of 60 to 90 min before addition of toxin.

Two protocols were used for exposing tissues to toxin. In the first, toxin was merely

added to normal physiological solution (33°C) and maintained for the duration of the experiment. The rate of spontaneous MEPPs was monitored throughout the experiment. In the second, the temperature of the bath was lowered to 7°C, after which toxin was added for 60 min. At the end of incubation, tissues were washed to remove unbound toxin, temperature was raised to 33°C, and recording of MEPPs was resumed.

Neuromuscular blockade was defined as a 90% reduction in the rate of spontaneous MEPPs. If blockade did not occur within 240 min, tissues were considered resistant to toxin at the concentration tested. Control experiments were done to demonstrate the viability of preparations in the absence of toxin.

Receptor binding studies. Binding of toxin to human brain membrane preparations was measured as previously described (Bakry *et al.*, 1991). Membrane preparations were obtained by homogenizing tissue in iced Tris•HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged for 10 min at 1,000 x g, and the resulting homogenate was resuspended in fresh buffer and recentrifuged for 45 min at 40,000 x g. The final pellet was resuspended in Tris•HCl buffer, as described above.

Iodinated ligand (1.0 nM), prepared as described below, was mixed with 5 μg of membrane protein in 0.1 ml of pH 7.4 buffer containing 50 mM Tris•HCl, 100 mM NaCl and 1 mg/ml bovine serum albumin. The binding reaction was done at 23°C for 60 min, which was the amount of time necessary to reach equilibrium. The reaction was terminated by centrifugation (15,000 x g; 2 min), after which the pellet was washed and recentrifuged. The amount of iodinated ligand associated with membranes was quantified, and the results were corrected for nonspecific binding.

Molecular biology studies. A human brain library (Human Brain 5'-stretch plus cDNA, Clonetech) was screened twice with an 800 base pair fragment of open reading frame obtained from an initial screening with rat syntaxin 1A cDNA (kindly provided by Dr. R. Scheller,

Stanford University). A cDNA clone, designated pcDNA.HS.2, was selected and analyzed by primer walking of both strands and found to have 2088 base pairs. Dideoxyterminator reaction chemistry was used for automated Tag cycle sequencing, and the results were confirmed by manual dideoxynucleotide sequencing (Sanger *et al.*, 1977). Additionally, the 5' end sequencing was confirmed by PCR applied to library cDNA's with sets of primers designed to overlap the cloning sites. A putative start codon was located at nucleotide 2, and the open reading frame (nucleotide positions 2 - 868) suggested a coding region containing 288 translated amino acids (GenBank accession No. U12918).

The cloned human gene for syntaxin 1A was expressed *in vitro* using a rabbit reticulocyte lysate preparation (Promega TnT Transcription and Translation System). The circular DNA was inserted into a pCR™II vector with a T7 promoter, and expression was carried out in the presence of ^{35}S-methionine according to the manufacturer's instructions. The resulting preparation was fractionated on a sodium dodecylsulfate polyacrylamide gel (15 percent) and submitted to autoradiography (48 hr; -20°C). The molecular weight of the expression product was deduced by comparison with a set of molecular weight standards.

Neurotoxins and antibodies. Botulinum neurotoxin types A, C and D were isolated as previously described (Simpson *et al.*, 1988). Samples of botulinum neurotoxin type C were also provided by Dr. Y. Kamata (University of Osaka Prefecture). Human pentavalent (ABCDE) immune globulin against botulinum neurotoxin was obtained from Connaught Laboratories.

Samples of botulinum neurotoxin were radioiodinated with Bolton-Hunter reagent. Purified toxin (100 μg) was mixed with 1 mCi (^{125}I)Bolton-Hunter reagent in 100 mM borate buffer, pH 8.0, for 30 min at room temperature. Iodinated toxin was separated from free iodine by fractionation on Sephadex G-50 columns. Preparations of labeled material typically had a specific activity of 600 to 900 Ci/mmol and a residual toxicity of 70 to 90 percent.

Expression of human substrates and proteolysis by botulinum toxin. Human syntaxin 1A was cloned and sequenced (see above and Results), and 1 μg of DNA for the gene was added to 25 μl of TNT Coupled Reticulocyte Lysate (Promega), 1 μl T7 RNA polymerase (per kit instructions), and 20 μCi ^{35}S-labeled methionine. The reaction mixture was incubated for 90 min at 30°C, after which it was centrifuged (~12,000 x g) for 15 min. The pellet was washed twice, then resuspended in proteolysis buffer (25 mM HEPES, pH 7.4; 50 mM NaCl; 10 μM $ZnCl_2$).

Human SNAP-25 was obtained by PCR, using template DNA from a human brain cDNA library (Human brain 5'-stretch plus cDNA; Clonetech). The primers were 5'-ATGGCCGAAGACGCAGAC-3' and 5'-GCACACTTAACCACTTCC-3', which cover the entire open reading frame (nucleotides 89-805; Bark and Wilson, 1994). The authenticity of the product was confirmed by sequencing. The PCR product was cloned into the TApCR vector (Invitrogen) and subcloned into the EcoRI site of Bluescript SK- (Stratagene). Human SNAP-25 was transcribed and translated *in vitro* with the TNT System and T3 RNA polymerase. The product was centrifuged, washed and suspended in proteolysis buffer, as described above.

In proteolysis experiments, botulinum neurotoxin types A and C were pre-reduced with 10 mM with DTT (45 min at 37°C). Toxin was then incubated with substrate for 60 min at 37°C. The reaction was terminated by boiling in sodium dodecylsulfate sample buffer for 3 min, then run on a polyacrylamide gel (12%). Dried gels were exposed to X-ray film for 16 hr. The molecular weights of substrates and reaction products were determined by comparison with standards.

RESULTS

Selection of tissues. A variety of innervated muscle preparations were evaluated, including tissues from the head and neck, trunk, and upper and lower limbs. Of the many preparations tested, the only one that proved suitable was the pyramidalis muscle (see Discussion). This tissue, which is located along the lower abdominal wall at the base of the rectus abdominus, is relatively small in size, surgically accessible, and reasonably available (see Discussion).

Characteristics of the human pyramidalis muscle preparation. Each muscle was probed with microelectrodes in an effort to localize endplate regions. Intracellular recordings in a large series of endplates (n=107) revealed an average resting membrane potential of -61.5 $\pm$ 0.7 mV. Resting potentials were well maintained for periods of 4 to 6 hours when tissues were kept at 33°C. Membrane potential and tissue responsiveness tended to diminish when experiments were done for comparable lengths of time at 37°C.

The rate of spontaneous MEPPs in physiological solution was 0.14 $\pm$ 0.03 per second (n=11; temperature, 33°C). This rate increased in a concentration-dependent manner with elevations in extracellular potassium. The mean amplitude of spontaneous MEPPs was 2.4 $\pm$ 0.08 mV (n=27), and the amplitude distribution was Gaussian in nature. The mean duration of spontaneous MEPPs was 3.4 $\pm$ 0.19 msec (n=24).

Characteristics of evoked responses. Surgically-excised preparations of human pyramidalis muscle frequently did not have a sufficient nerve stump to permit direct, microelectrode-induced depolarization. Therefore, mild potassium-induced depolarization was used as an alternative. In a typical experiment, tissues were maintained in 12.5 mM potassium for a baseline period of 30 to 60 min, and the frequency of MEPPs was monitored. Under these conditions, the baseline rate of MEPPs was 1.5 $\pm$ 0.12 per second (n=50). At the end

of the baseline period, tissues were transiently exposed to 25 mM potassium. This caused the rate of MEPPs to increase by approximately one order of magnitude (14.5 $\pm$ 2.7 per sec). When tissues were reimmersed in 12.5 mM potassium, the rate of MEPPs fell to baseline levels.

Human pyramidalis muscle responses to 12.5 mM and 25 mM potassium were well maintained for extended periods of time. When immersed in 12.5 mM potassium solution, tissues displayed a stable MEPP rate for periods of 240 to 300 min. When tissues were briefly exposed to 25 mM potassium at intervals of approximately 60 min, the magnitudes of responses were comparable (see below). These stable responses permitted an analysis of botulinum neurotoxin action.

Botulinum neurotoxin type A action. The addition of botulinum neurotoxin type A to human pyramidalis muscle preparations produced irreversible blockade of transmission. This could be demonstrated by using two different paradigms and two different measures of outcome. When toxin (1×10^{-8} M) was added to tissues maintained at 33°C in 12.5 mM potassium solution, the baseline rate of MEPPs remained constant for about 30 to 40 min and then began to decay. Within 60 to 90 min the baseline rate of MEPPs fell to levels that were too low to measure. This evidence of toxin-induced blockade was confirmed by immersing tissues in 25 mM potassium solution. The spike in MEPP frequency ordinarily associated with high potassium solution was almost completely abolished.

A second paradigm generated closely similar results. Tissues were initially immersed in medium at 33°C, and both baseline responses to 12.5 mM potassium and evoked responses to 25 mM potassium were monitored. Tissues were then transferred to physiological solution at 7°C, after which toxin was added (1×10^{-8} M) for an incubation period of 60 min. Following incubation, tissues were washed to remove unbound toxin, temperature was raised to 33°C, and MEPP frequency was monitored in 12.5 mM potassium solution. As before, the toxin produced an irreversible decay in the rate of MEPPs. Also as before, the normal response to

elevated potassium (25 mM) was virtually abolished.

Botulinum neurotoxin type C action. An identical series of experiments was performed with botulinum toxin type C (1×10^{-8} M). Quite unexpectedly, this serotype also produced blockade of neuromuscular transmission. Regardless of whether toxin was present continuously at 33°C or present for only 60 min at 7°C, the sequence of events was the same. There was an initial lag period, after which the baseline rate of MEPPs in 12.5 mM potassium decayed and eventually became almost unmeasurable.

To ensure that the observed response was authentic and could be attributed to serotype C, two control experiments were done. In the first, the neurotoxin was isolated for a second time from a different strain of *Clostridium botulinum*. This batch of toxin, like the original serotype C, produced neuromuscular blockade. In the second experiment, serotype C was preincubated with neutralizing antibody before being added to tissues. In this case there was no neuromuscular blockade.

Botulinum neurotoxin type D action. At concentrations equivalent to those tested with serotypes A and C, botulinum neurotoxin type D (1×10^{-8} M) did not block human neuromuscular transmission (n=3). The rate of spontaneous and evoked MEPPs remained stable in the presence of toxin for periods up to four hours. Even when toxin concentration was increased an order of magnitude, there was still no evidence of paralysis. These results indicate that the human neuromuscular junction is resistant to serotype D.

Binding of botulinum toxin to human nerve cell membranes. The fact that botulinum toxin types A and C blocked transmission implies that the human nervous system has cell surface receptors. Therefore, work was done to verify the existence of these receptors and to characterize toxin~receptor interactions.

Preliminary experiments with serotypes A and C and membrane preparations from several

areas of human brain (prefrontal cortex, anterior temporal cortex, superior parietal cortex, putamen, globus pallidus, and cerebellum) revealed that the cerebellum typically had the highest density of toxin binding sites. Therefore, this region of the human nervous system was examined in some detail.

The binding of ^{125}I-botulinum neurotoxin type A to human cerebellar membranes increased as a function of protein; an apparent plateau was reached at 100 to 200 μg/assay (100 μl/assay). Binding also increased as a function of time, with an apparent equilibrium being reached at 15 to 20 min.

Various concentrations of iodinated botulinum neurotoxin were incubated with membrane preparations (50 μg/ml protein; 60 min), and the resulting data were used to generate a saturation isotherm and a Scatchard plot. Graphic analysis of the data revealed two classes of binding sites. The characteristics of the high affinity binding site were: Kd, 0.3 nM; Bmax, 0.78 pmol/mg protein. The characteristics of the low affinity site were: Kd 3.3 nM; Bmax, 3.18 pmol/mg protein. In related experiments, various concentrations of unlabeled serotype A were used as competitive antagonists of the binding of labeled serotype A. The apparent IC50 for the unlabeled toxin, as deduced by graphic analysis, was 3.5 nM, which is in agreement with the Kd value for the bulk of binding sites (e.g., low affinity sites) in these membranes. By contrast, a substantial molar excess (0.3 μM) of heterologous toxin (i.e., serotype C) did not antagonize the binding of iodinated type A (and see below).

The binding of botulinum neurotoxin type C to nerve membranes was studied under conditions equivalent to those used with serotype A. The results indicated the presence of specific binding sites; toxin association with these binding sites increased as a function of time (equilibrium ca 30 to 60 min) and protein (half maximal binding ca 50 to 100 μg/ml).

Various concentrations of iodinated botulinum toxin type C were incubated with nerve membranes (50 μg/ml; 60 min), and the resulting data on specific binding were transformed into a Scatchard plot. Interestingly, graphic analysis of the data yielded a finding that was different from that obtained with serotype A. The work demonstrated the existence of a single

class of binding sites whose characteristics were: Kd = 1.96 ± 0.36 nM; Bmax = 8.9 ± 0.56 pmol/mg protein. Specific binding of iodinated serotype C could be blocked by unlabeled type C, but not by unlabeled type A.

Human substrates for botulinum neurotoxin. The principal substrate for serotype A is SNAP-25 (Blasi *et al.*, 1993a; Schiavo *et al.*, 1993a), which has previously been cloned and sequenced (Bark and Wilson, 1994). The substrates for serotype C are syntaxin (Blasi *et al.*, 1993b), for which the human gene has not been cloned and sequenced, and SNAP-25 (Williamson *et al.*, 1996; Foran *et al.*, 1996).

A gene for human syntaxin was isolated, cloned and sequenced, as described under Methods. The deduced amino acid sequence of the open reading frame revealed a polypeptide that was strikingly similar in length and primary sequence to rat syntaxin 1A (Zhang *et al.*, 1995). The overall identity in amino acid sequences between the two syntaxins was approximately 98%. Therefore, the expression product was deduced to be human syntaxin 1A.

Cleavage of human substrates. SNAP-25 and syntaxin 1A were expressed *in vitro*, as described under Methods. Serotypes A and C, each at 1 x 10^{-7} M, were reduced with dithiothreitol (10 mM; 37°C; 30 min), then incubated with substrates (37°C; 60 min). The reaction mixtures were submitted to polyacrylamide gel electrophoresis (12%), and the dried gels were subsequently exposed to X-ray film for 16 hr.

Serotypes A and C caused proteolytic cleavage of their respective human substrates. Furthermore, cleavage was in keeping with the zinc-dependent endoprotease action of the toxins. Thus, incubation of toxin and substrate in the presence of a zinc chelator [50 μM tetrakis(2-pyridylmethyl)ethylenediamine] markedly diminished proteolysis of SNAP-25 and syntaxin 1A.

DISCUSSION

The literature describing electrophysiological properties of excised human neuromuscular junctions is very limited. This is due in large measure to ethical constraints, which properly restrict the circumstances under which normal tissue can be removed from patients. One possible remedy that respects the ethical constraints is to harvest tissue that would ordinarily be removed or be damaged *in situ* during routine surgical procedures. However, this brings to light a host of experimental concerns. To be acceptable as an experimental preparation, a human tissue should possess the following characteristics: *1)* reasonable availability, based on accessibility of muscle during various surgical procedures, *2)* reasonable consistency in size, *3)* ease of orientation after removal from patients, *4)* ease of localization of endplate regions, and *5)* ability to survive and respond for substantial periods of time. These criteria were best satisfied by the pyramidalis muscle, which has one additional advantage (Chouke, 1935; Beaton and Anson, 1939; Monkhouse and Khalique, 1986). The muscle is generally regarded as non-essential, and thus partial or complete removal does not impair the donor.

Electrophysiologic studies of the pyramidalis muscle revealed that the endplate region was localized to discrete areas. Intracellular recordings at the endplate region demonstrated a resting membrane potential of -61.5 mV. This was well maintained over 4 to 6 hours at a constant temperature of 33°C. The rate of MEPPs in physiological solution was 0.14 per second, and this value is in keeping with that previously reported for other human neuromuscular junctions (Elmqvist and Quastel, 1965; Lambert and Elmqvist, 1971; Haynes, 1971; Maselli *et al.*, 1991; Maselli *et al.*, 1992; Slater *et al.*, 1992). The amplitude distribution of spontaneous MEPPs was consistent with a Gaussian distribution.

Elevations in extracellular potassium produced concentration-dependent increases in the rate of MEPPs. These evoked increases in MEPP rate, like spontaneous MEPP rate, were well maintained over time. This afforded the opportunity to examine botulinum neurotoxin action.

Mechanism of toxin action. Botulinum toxin proceeds through a series of steps to produce its effects on cholinergic nerve endings. This includes binding, productive internalization, and eventual expression of an intracellular effect (see Introduction for references). This general scheme for describing toxin action arose from studies on the murine phrenic nerve-hemidiaphragm preparation (Simpson, 1980), and with few exceptions (e.g., Apylsia; Poulain *et al.,* 1989; Poulain *et al.,* 1991), it has proved useful for describing toxin action on other cholinergic junctions. However, there is no way to know whether this model applies to the human neuromuscular junction, because there have been no studies on isolated human tissues. Therefore, one of the goals of this work was to analyze toxin action on excised human neuromuscular junctions. Serotypes A, C and D were selected for study.

Botulinum neurotoxin type A produced irreversible blockade of transmission in the pyramidalis preparation. This could be demonstrated by using a standard paradigm in which toxin was continuously present, and it could also be shown using a binding paradigm in which the toxin was present for only a limited time. The latter approach arose from studies on murine preparations (Simpson, 1980). When mouse phrenic nerve-hemidiaphragms are incubated with toxin at a low temperature ($< 10°C$), the toxin binds to the tissue but does not cause paralysis. The absence of blockade is due to the fact that receptor-mediated endocytosis is arrested at low temperature, and thus toxin is not internalized. Transmission does not begin to fail until temperature is elevated.

The actions of botulinum neurotoxin type A on human tissues were entirely consistent with this model. When toxin was added to tissues at 7°C, there was no evidence for onset of paralysis. When tissues were washed and then suspended in toxin-free medium at 33°C, there was a lag time that could account for internalization and then onset of blockade. Paralysis of transmission was demonstrated in two ways: *1)* the rate of spontaneous MEPPs fell more than 90%, and *2)* the sharp rise in MEPP rate due to elevated potassium was nearly abolished.

Botulinum neurotoxin types C and D were also studied on the human pyramidalis preparation. In contrast to serotype C, which appeared to block transmission similarly to

serotype A, serotype D produced no observable effect. Even when used at concentrations 10-fold higher than with the other two serotypes, type D still produced no measurable effect over a period of 4 to 5 hours.

The finding that serotype D does not poison human preparations is in keeping with epidemiologic data. There has never been a confirmed case of type D human botulism. On the other hand, the finding that serotype C blocked transmission was unexpected. There are no confirmed cases of type C poisoning in adults, and there is only one report of type C poisoning in a single infant (Oguma *et al.*, 1990). In view of the apparent difference between epidemiologic data and isolated tissue data, a number of experiments were done to assure that type C does indeed act on human tissues. This included: *1)* isolation and testing of type C toxin from two different strains of clostridia, *2)* neutralization of toxicity with specific antibodies, *3)* demonstration that the human nervous system has high affinity binding sites for serotype C, *4)* demonstration that the human nervous system has a gene encoding syntaxin 1A, the major substrate for serotype C, and *5)* demonstration that serotype C cleaves the translation product of the human syntaxin 1A gene. In the aggregate, this is compelling evidence that isolated human tissues are susceptible to the toxin.

The fact that type C toxin does paralyze human neuromuscular transmission naturally raises the question why the toxin does not typically cause human botulism. Although there could be many possible explanations, two are particularly obvious. Apparent resistance may be due to ecological factors (i.e., lack of human exposure) or to physiological factors (i.e., poor human absorption). Experiments are currently in progress in an effort to answer this question.

Therapeutic Issues. The data presented here bear on three interrelated issues of patient care: *1)* the appropriateness of developing and administering a polyvalent vaccine against botulinum neurotoxin, *2)* the lack of appropriateness of including serotype C in any vaccine formulation, and *3)* the need to evaluate serotype C as a therapeutic agent for dystonia.

There currently exist a number of experimental vaccines against botulinum toxin, including a pentavalent vaccine (A, B, C, D and E) distributed by the Centers for Disease Control. These vaccines were developed and were being administered long before it was realized that botulinum toxin has value as a therapeutic agent. As a result, there are vaccinated persons who, should they develop dystonia, would be unresponsive to botulinum toxin therapy. This is a serious matter given that: *1)* botulinum toxin is the only therapeutic intervention that gives satisfactory results for most patients with dystonia, and *2)* vaccination can produce long-term resistance to toxin, i.e., a decade or longer. These facts argue strongly that one must be cautious and thoughtful about administering vaccine.

This argument takes on added weight when viewed in the context of recent immunology work. The complete primary structures of the botulinum serotypes have been determined, and all possess similarity to tetanus toxin. Recombinant molecular biology techniques have been used to generate experimental vaccines to tetanus (Fairweather *et al.*, 1987; Chatfield *et al.*, 1992; Boucher *et al.*, 1994), and the same will likely occur for botulinum toxin. This creates the temptation to develop and administer a polyvalent vaccine against botulism and tetanus. However, this is a course of action that may not be in the best interest of patient care. The incidence of naturally-occurring botulism is orders of magnitude less than the incidence of dystonia. If a polyvalent vaccine against botulinum toxin were to be widely administered, it would produce the negligible benefit of protecting against botulism but at the same time produce the substantial risk of causing all current and future dystonia patients to become unresponsive to the only medication that has authentic value. This risk-to-benefit analysis suggests that immunization should be provided only when there is an identifiable and meaningful clinical gain.

On a related issue, there is strong reason to argue that serotype C should not be included in any vaccine. Although the toxin does act on isolated human tissues, this does not challenge or diminish the observation that type C botulism rarely if ever occurs in adults. The explanation for the apparent absence of disease may relate to ecological factors or

physiological factors, as discussed earlier. Whatever the explanation, the low incidence of type C botulism argues against the need for a vaccine.

On a more positive note, the data encourage the testing of serotype C as a therapeutic agent for dystonia and related neurologic disorders. In the past, the epidemiologic literature on naturally occurring botulism was used as a guide in selecting toxin serotypes to test as medicinal agents. Serotype A has long been implicated in human illness, it was the first serotype to be evaluated as a medicinal agent, and it is the only serotype that has been approved for clinical use (Jankovic and Brin, 1991). Other serotypes implicated in human illness (B, E and F) are now undergoing clinical trials (Jankovic and Hallett, 1994). However, the finding that serotype C blocks human neuromuscular transmission means that the practice of relying solely on epidemiology as a guide for choosing therapeutic agents is flawed. Clearly, serotype C warrants investigation for the treatment of neurologic disorders.

The possibility that serotype C is efficacious carries a hidden benefit. If a situation were to arise in which it was necessary to provide immunization against naturally occurring botulism, there would be no need to include serotype C in the vaccine. This would mean that it should be possible to protect patients against food-borne botulism (e.g., serotype A) while not depriving them of the ability to respond to anti-dystonia medication (e.g., serotype C).

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Dr. Hee-Ok Park for suggesting the pyramidalis muscle as one of the human preparations that should be evaluated.

REFERENCES

American Academy of Neurology: Assessment: the clinical usefulness of botulinum toxin-A in treating neurological disorders. Neurology **40**: 1332-1336, 1990.

Bakry, N., Kamata, Y., Sorensen, R. and Simpson, L. L. Tetanus toxin and neuronal membranes: the relationship between binding and toxicity. J. Pharmacol. Exp. Ther. **258**: 613-619, 1991.

Bark, I. C. and Wilson, M. C. Human cDNA clones encoding two different isoforms of the nerve terminal protein SNAP-25. Gene **139**: 291-292, 1994.

Beaton, L. E. and Anson, B. J. Pyramidalis muscle: its occurrence and size in American whites and Negroes. Am. J. Phys. Anthrop. **25**: 261-269, 1939.

Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Sudhof, T. C., Jahn, R. and Niemann, H. Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J. Biol. Chem. **269**: 1617-1620, 1994.

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Südhof, T. C., Niemann, H. and Jahn, R. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature **365**: 160-163, 1993a.

Blasi, J., Chapman, E. R., Yamasaki, S., Binz, T., Niemann, H. and Jahn, R. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO J. **12**: 4821-4828, 1993b.

Boucher, P., Sato, H., Sato, Y. and Locht, C. Neutralizing antibodies and immunoprotection against pertussis and tetanus obtained by use of a recombinant pertussis toxin-tetanus toxin fusion protein. Infect. Immun. **62**: 449-456, 1994.

Chatfield, S. N., Charles, I. G. and Makoff, A. J. Use of the nirB promotor to direct the stable expression of heterologous antigens in salmonella oral vaccine strains: development of a single dose oral tetanus vaccine. Bio/Technology **10**: 888-892, 1992.

Chouke, K. S. The constitution of the sheath of the rectus abdominus muscle. Anat. Rec. **61**: 341-349, 1935.

Dowell, V. R.,Jr. Botulism and tetanus: selected epidemiologic and microbiologic aspects. Rev. Infect. Dis. **6 Suppl 1**: S202-S207, 1984.

Elmqvist, D. and Quastel, D. M. A quantitative study of end-plate potentials in isolate human muscle. J. Physiol. **178**: 505-529, 1965.

Fairweather, N. F., Lyness, V. A. and Maskell, D. J. Immunization of mice against tetanus with fragments of tetanus toxin synthesized in *Escherichia coli*. Infect. Immun. **55**: 2541-2545, 1987.

Foran, P., Lawrence, G. W., Shone, C. C., Foster, K. A. and Dolly, J. O. Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. Biochem. **35**: 2630-2636, 1996.

Gangarosa, E. J., Donadio, J. A., Armstrong, R. W., Meyer, K. F., Brachman, P. S. and Dowell, V. R. Botulism in the United States, 1899-1969. Am. J. Epidemiol. **93**: 93-101, 1971.

Habermann, E. and Dreyer, F. Clostridial neurotoxins: Handling and action at the cellular level. Curr. Topics Microbiol. Immunol. **129**: 93-179, 1986.

Haynes, J. Miniature end-plate potentials in neuromuscular disease: an electrophysiological investigation of motor-point muscle biopsies. J. Neurol. Neurosurg. Psychiat. **34**: 521-526, 1971.

Jankovic, J. and Brin, M. F. Therapeutic uses of botulinum toxin. N. Engl. J. Med. **324**: 1186-1194, 1991.

Jankovic, J. and Hallett, M. Therapy with Botulinum Toxin, Marcel Dekker, Inc. New York, 1994.

Lambert, E. H. and Elmqvist, D. Quantal components of end-plate potentials in the myasthenic syndrome. Ann N. Y. Acad. Sci. **183**: 183-199, 1971.

Maselli, R. A., Mass, D. P., Distad, B. J. and Richman, D. P. Anconeus muscle: a human muscle preparation suitable for in vitro microelectrode studies. Muscle & Nerve **14**: 1189-1192, 1991.

Maselli, R. A., Burnett, M. E. and Tonsgard, J. H. In vitro microelectrode study of neuromuscular transmission in a case of botulism. Muscle & Nerve **15**: 273-276, 1992.

Monkhouse, W. S. and Khalique, A. Variations in the composition of the human rectus sheath: a study of the anterior abdominal wall. J. Anat. **145**: 61-66, 1986.

NIH Consensus Statement: Clinical use of botulinum toxin. Arch. Neurol. **48**: 1294-1298, 1991.

Oguma, K., Yokota, K., Hayashi, S., Takeshi, K., Kumagai, M., Itoh, N., Tachi, N. and Chiba, S. Infant botulism due to *Clostridium botulinum* type C toxin [letter]. Lancet **336**: 1449-1450, 1990.

Pevsner, J., Hsu, S. C., Braun, J. E. A., Calakos, N., Ting, A. E., Bennett, M. K. and Scheller, R. H. Specificity and regulation of a synaptic vesicle docking complex. Neuron **13**: 353-361, 1994.

Poulain, B., Wadsworth, J. D. F., Shone, C. C., Mochida, S., Lande, S., Melling, J., Dolly, J. O. and Tauc, L. Multiple domains of botulinum neurotoxin contribute to its inhibition of transmitter release in Aplysia neurons. J. Biol. Chem. **264**: 21928-21933, 1989.

Poulain, B., Mochida, S., Weller, U., Högy, B., Habermann, E., Wadsworth, J. D. F., Shone, C. C., Dolly, J. O. and Tauc, L. Heterologous combinations of heavy and light chains from botulinum neurotoxin A and tetanus toxin inhibit neurotransmitter release in Aplysia. J. Biol. Chem. **266**: 9580-9585, 1991.

Sanger F., Nicklen S. and Coulson A. R. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**: 5463-5467, 1977.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. R. and Montecucco, C. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature **359**: 832-835, 1992.

Schiavo, G., Rossetto, O., Catsicas, S., de Laureto, P. P., DasGupta, B. R., Benfenati, F. and Montecucco, C. Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D and E. J. Biol. Chem. **268**: 23784-23787, 1993a.

Schiavo, G., Shone, C. C., Rossetto, O., Alexander, F. C. G. and Montecucco, C. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. J. Biol. Chem. **268**: 11516-11519, 1993b.

Schiavo, G., Malizio, C., Trimble, W. S., Polverino de Laureto, P., Sugiyama, H., Johnson, E. A. and Montecucco, C. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. J. Biol. Chem. **269**: 20213-20216, 1994a.

Schiavo, G., Rossetto, O. and Montecucco, C. Clostridial neurotoxins as tools to investigate the molecular events of neurotransmitter release. Seminars in Cell Biology **5**: 221-229, 1994b.

Simpson, L. L. Kinetic studies on the interaction between botulinum toxin type A and the cholinergic neuromuscular junction. J. Pharmacol. Exp. Ther. **212**: 16-21, 1980.

Simpson, L. L. The origin, structure, and pharmacological activity of botulinum toxin. Pharmacol. Rev. **33**: 155-188, 1981.

Simpson, L. L. Botulinum Neurotoxin and Tetanus Toxin, Academic Press, San Diego, 1989.

Simpson, L. L., Schmidt, J. J. and Middlebrook, J. L. Isolation and characterization of the botulinum neurotoxins. Methods Enzymol. **165**: 76-85, 1988.

Slater, C. R., Lyons, P. R., Walls, T. J., Faqcett, P. R. W. and Young, C. Structure and function of neuromuscular junctions in the vastus lateralis of man. Brain **115**: 451-478, 1992.

Söllner, T., Bennett, M. K., Whitehead, S. W., Scheller, R. H. and Rothman, J. A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell **75**: 409-418, 1993a.

Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Germanos, S., Tempst, P. and Rothman, J. SNAP receptors implicated in vesicle targeting and fusion. Nature **362**: 318-324, 1993b.

Südhof, T. C., De Camilli, P., Niemann, H. and Jahn, R. Membrane fusion machinery: insights from synaptic proteins. Cell **75**: 1-4, 1993.

Williamson, L. C., Halpern, J. L., Montecucco, C., Brown, J. E. and Neale, E. A. Clostridial neurotoxins and substrate proteolysis in intact neurons. J. Biol. Chem. **271**: 7694-7699, 1996.

Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., Roques, B., Fykse, E. M., Südhof, T. C., Jahn, R. and Niemann, H. Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. J. Biol. Chem. **269**: 12764-12772, 1994a.

Yamasaki, S., Binz, T., Hayashi, T., Szabo, E., Yamasaki, N., Eklund, M., Jahn, R. and Niemann, H. Botulinum neurotoxin type G proteolyses the Ala81-Ala82 bond of rat synaptobrevin 2. Biochem. and Biophys. Res. Comm. **220**: 829-835, 1994b.

Zhang, R., Maksymowych, A. B. and Simpson, L. L. Cloning and sequence analysis of a cDNA encoding human syntaxin 1A, a polypeptide essential for exocytosis. Gene **159**: 293-294, 1995.

SECTION 2

BINDING OF BOTULINUM NEUROTOXIN TO NERVE MEMBRANES OF HUMAN AND NONHUMAN ORIGIN

ABSTRACT

Iodinated botulinum neurotoxin type B binding to nerve membranes was studied on mouse, rat and human preparations. The toxin was examined both in the single chain and in the proteolytically processed dichain form, and binding sites were assayed both in spinal cord and in various brain regions. Mouse, rat and human brain possessed specific binding site for botulinum neurotoxin type B. The average Kd values for the various membrane preparations examined were: mouse, 3.7 $\pm$ 0.9 nM; rat, 4.2 $\pm$ 0.7 nM; human, 18.2 $\pm$ 5.0 nM. The binding of botulinum toxin type B to rat brain membranes was not antagonized by a polyclonal antibody against the cytosolic domain of synaptotagmin or by a monoclonal antibody directed against the lumenal domain of synaptotagmin. In addition, these antibodies did not protect the mouse phrenic nerve-hemidiaphragm from toxin-induced neuromuscular blockade. A comparison of tissues from non-Alzheimer's disease patients and Alzheimer's disease patients revealed that the latter had a reduction in the number of specific binding sites. This reduction, which was most pronounced in cerebellum, putamen and globus pallidus, was likely due to a reduction in Bmax. Extraction of whole brain mRNA and injection into Xenopus oocytes led to expression of binding sites for botulinum neurotoxin. Extraction and injection of cerebellar mRNA led to expression of a higher density of binding sites. The number of binding sites was not diminished when oocytes were pretreated with antibodies against the cytosolic and lumenal domains of synaptotagmin. These findings are likely to aid in the isolation, characterization and reconstitution of toxin binding sites.

INTRODUCTION

Botulinum neurotoxin is a large protein synthesized and released by the organism *Clostridium botulinum*. The toxin exists in seven different serotypes, designated A, B, C1, D, E, F and G, but each of these serotypes has a similar structure and mechanism of action (for review of toxin structure, see DasGupta, 1989). In the immediate post-translational stage, botulinum neurotoxin is a single chain polypeptide with a molecular weight of a ca. 150,000. Some organisms possess an endogenous protease that cleaves the neurotoxin to yield a dichain molecule (heavy chain~100,000; light chain~50,000) with an interchain disulfide bond. In the absence of endogenous protease, the neurotoxin can be cleaved by exogenous proteases such as trypsin. Proteolytic processing is essential for the neurotoxin to attain full biological activity.

All serotypes of botulinum neurotoxin act preferentially on cholinergic nerve endings to block acetylcholine release (for review of mechanism of toxin action, see Habermann and Dreyer, 1986; Simpson, 1989; 1993). The toxin produces this effect by proceeding through an elaborate series of steps, the first of which involves toxin binding to receptors on the surface of nerve membranes. The identity of these receptors and their role in nerve structure or function have not been determined. The next step in the sequence is productive internalization, which in reality is a combination of two events. The toxin penetrates the cell membrane by receptor-mediated endocytosis, and it then penetrates the endosome membrane by pH-induced translocation. Toxin that reaches the cytosol acts enzymatically to cleave synaptobrevin (Schiavo *et al.*, 1992; 1993a; 1993b; 1994; Yamasaki *et al.*, 1994a; 1994b), SNAP-25 (Blasi *et al.*, 1993a; Schiavo *et al.*, 1993a; Binz *et al.*, 1994) or syntaxin (Blasi *et al.*, 1993b), which are polypeptides that are essential for docking and fusion of vesicles. Cleavage of these polypeptides culminates in blockade of exocytosis.

Of the various steps involved in toxin-induced blockade of transmitter release, the binding step is perhaps the least well understood. There are relatively few studies that have been done

to characterize toxin binding to nerve membranes (e.g., kinetic analyses of toxin binding to brain membrane preparations), and even fewer studies that have been done to isolate the toxin receptor (for reviews, see Middlebrook, 1989; Montecucco and Schiavo, 1994). As a result, the receptor has not yet been identified, and there are certainly no data that characterize molecular interactions between toxin and receptor. This contrasts sharply with knowledge about intracellular actions of the toxin. For the latter, at least a portion of the catalytic domain in the toxin has been localized, the substrate for the toxin has been identified, and the amino acid residues that form the toxin cleavage site have been determined (Schiavo *et al.*, 1992; 1993a; 1993b; 1994; Blasi *et al.*, 1993a; 1993b; Binz *et al.*, 1994; Yamasaki *et al.*, 1994a; 1994b).

In the work that follows, an effort has been made to begin the process of characterizing botulinum neurotoxin binding sites on nerve membrane preparations. Botulinum neurotoxin type B was selected for these studies, and the molecule was examined both in the single chain and dichain form. The work was intended to address three interrelated issues, as follows: *1)* determine which species of laboratory animals possess toxin binding sites, and compare the extent of binding in different brain regions, *2)* measure toxin binding in different regions of human brain, and compare the extent of this binding in normal tissue and in diseased tissue, and *3)* attempt to induce toxin binding sites by transferring brain mRNA to a model expression system.

MATERIALS AND METHODS

Botulinum neurotoxin. Botulinum neurotoxin was isolated and purified as previously described (Sakaguchi, 1982; Simpson *et al.*, 1988). The toxin was extracted from young cultures that are free of proteases that nick the molecule, and thus the toxin was isolated in the single chain form. Conversion of the toxin to the dichain form was achieved by adding it to N-tosyl-phenylalanine chloromethylketone-treated trypsin that was coupled to agarose beads [trypsin:toxin, 1:40 (w:w)]. The mixture was incubated at 37°C for 15 min in 0.02 M sodium phosphate buffer, pH 7.0. The reaction was terminated by centrifugation and later aspiration of activated toxin. The homogeneity and molecular structure of the toxin were confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The biological activity of the unactivated and activated species of the toxin was assayed on phrenic nerve hemidiaphragm preparations (see below).

Botulinum neurotoxin type B was radioiodinated with the Bolton-Hunter reagent. Purified toxin (100 μg) was mixed with 1 mCi of (^{125}I)Bolton-Hunter reagent in 100 mM borate buffer, pH 8.0, for 30 min at room temperature. Iodinated toxin was separated from free iodine by fractionation of the reaction mixture on a Sephadex G-50 column. Various preparations of the labeled material had a specific activity of 600 to 900 Ci/mmol and a residual toxicity of 70 to 90 percent.

Neuromuscular preparations. Mouse phrenic nerve hemidiaphragm preparations were excised and used as previously described (Coffield *et al.*, 1994). Tissues were immersed in physiological solution of the following composition (mM): NaCl, 137; KCl, 5.0; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; $NaHPO_4$, 1.0 and d-glucose, 11. The solution was bubbled with 95% O_2, 5% CO_2, and it contained gelatin (0.01%) as an auxiliary protein to diminish nonspecific inactivation of toxin.

When tissues were used to bioassay the activity of toxin preparations, tissues were

immediately immersed in medium at 35°C. Various concentrations of toxin were added (see Results), after which phrenic nerves were stimulated (0.5 Hz) and the amount of time necessary for development of paralysis was monitored. When tissues were used to detect any possible interaction between synaptotagmin antibodies and toxin, they were immersed in medium at 5°C. Antibody was added for 30 min, after which toxin was added for an additional 30 min (in the continuing presence of antibody). At the end of the 60 min incubation period, tissues were washed and suspended in antibody-free, toxin-free solution at 35°C, and paralysis was monitored as above. Neuromuscular blockade was defined as a 90% reduction in muscle twitch evoked by nerve stimulation.

Nerve membrane preparations. Brain and spinal cord were obtained from mice and rats. Tissues were removed according to protocols approved by the Institutional Animal Care and Use Committee. Brain tissue was also obtained at autopsy from human patients with no known neurological disorders and from patients with Alzheimer's disease. This tissue was removed and used under a protocol approved by the Institutional Review Board. All experiments were conducted in BL-2 or BL-3 facilities, and all procedures were in keeping with institutional and federal guidelines that pertain to potential exposure of investigators to blood-borne pathogens.

Nerve membrane preparations were obtained by homogenizing brain or spinal cord in iced Tris · HCl buffer (50 mM, pH 7.4). The homogenate was centrifuged for 10 min at 1,000 x g, and the resulting homogenate was resuspended in fresh buffer and recentrifuged for 45 min at 40,000 x g. The final pellet was resuspended in Tris · HCl buffer (as above).

Xenopus oocytes. Oocytes were obtained from adult female *Xenopus laevis* (African clawed toad). One lobe of the ovary was surgically excised from anesthetized toads (0.2% aqueous solution of 3-aminobenzoic acid ethyl ester methanesulfonate salt), and oocytes were separated by incubation in calcium-free saline (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 5

mM HEPES; pH 7.6) containing 2 mg/ml collagenase A. Defolliculated oocytes were maintained in sterile solution (50% Leibovitz's solution containing 5 mM glutamine and 15 mM HEPES at pH 7.6, plus 100 mg/ml Gentamycin) at 18°C for at least 8 hr before being used. Oocytes were then microinjected with mRNA (75 to 100 ng/oocyte) and incubated for another 2 to 3 days. Oocyte membranes were prepared similarly to brain membranes, except that the original centrifugation was at 300 x g and the subsequent centrifugation was at 30,000 x g.

Ligand binding experiments. The binding of iodinated botulinum neurotoxin type B to brain, spinal cord or oocyte membranes was measured by a centrifugation assay as previously described (Bakry *et al.*, 1991a; 1991b). The ligand was mixed with a specified concentration of membrane (see Results) in 100 μl of buffer containing 50 mM Tris · HCl, pH 7.4, 100 mM NaCl and 1 mg/ml of bovine serum albumin. The binding reaction was allowed to proceed at room temperature (ca. 23°C) for the amount of time necessary to reach equilibrium (see Results). The reaction was terminated by centrifugation (15,000 x g, 2 min), after which the pellet was washed with fresh buffer and recentrifuged. Membrane-associated ligand was collected and quantified, and the results were corrected for nonspecific binding. The data were evaluated by using the equilibrium binding analysis program of McPherson (1982).

Isolation of mRNA. Total RNA was extracted and isolated by the RNAzol B method, essentially as described in the product literature (BIOTECX Laboratories, Inc.; Houston, TX). Brain tissue was homogenized in RNAzol B solution with a Brinkmann Polytron®. The homogenate was mixed with chloroform (10 percent by volume) and vigorously shaken for 15 sec. The suspension was centrifuged at 12,000 x g for 15 min, after which the colorless aqueous phase was collected. Total RNA was precipitated by adding an equal volume of isopropanol (0°C, 30 min). Precipitated total RNA was collected and washed once with 70 percent ethanol. mRNA (poly "A" RNA) was separated from total RNA by the Expresep

method, as described in the product literature (BIOTECX Laboratories, Inc.). Isolated mRNA was dissolved in diethylpyrocarbonate-treated water and stored at -80°C.

RESULTS

Characterization of ligand. Botulinum neurotoxin type B isolated as previously described (Simpson *et al.*, 1988) was collected from a single peak at the conclusion of a series of chromatographic steps. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate confirmed that the isolated toxin was essentially homogeneous and in the single chain form. Proteolytic processing of the toxin with agarose-bound trypsin resulted in almost complete conversion to the dichain form.

The relative potencies of the single chain and dichain species were bioassayed on the mouse phrenic nerve-hemidiaphragm preparation, as previously described (Simpson and DasGupta, 1983). Proteolytic processing of the toxin resulted in an increase in potency of more than an order of magnitude.

Single chain and dichain forms of the toxin were radioiodinated, as described under Methods, to give preparations with high specific activity (range, 600 to 900 Ci/mmol). Labeled material was bioassayed for residual toxicity, as described above, and was found to retain between 70 and 90 percent of the activity of native toxin.

Radiolabeled toxin was submitted to polyacrylamide gel electrophoresis, and individual lanes containing the single chain and dichain forms of the toxin were excised and cut into 2 mm strips. As expected, lanes containing the single chain species had a single peak of radioactivity, and this peak migrated in a manner consistent with a molecular weight of ~150,000. Lanes containing the dichain species had two major peaks, and the molecular weights were consistent with those of the heavy chain (~95,000 to 100,000) and light chain (~51,000) of botulinum neurotoxin. Interestingly, the fraction of radioactivity associated with the heavy chain (~78%) was greater than that predicted on the basis of molecular weight. This means that radioactivity was enriched in that portion of the toxin molecule thought to mediate binding.

Binding of toxin to nerve membrane preparations. In an initial series of experiments, membrane preparations from mouse (brain), rat (brain), human (brain), and oocytes (whole cells) were tested for their ability to bind iodinated dichain toxin. Standard conditions for this binding assay were: toxin concentration, 0.5 to 1.0 nM; membrane concentration, 100 μg/assay; time, 2 hr; and temperature, 23°C. These conditions were similar to those previously used to study clostridial toxin binding to brain membrane preparations (Bakry *et al.*, 1991a; 1991b).

These preliminary experiments indicated that all tissues of mammalian origin displayed binding sites for toxin. Kinetic analyses and regional binding data are presented below. In contrast, native oocyte membranes had no specific binding sites for toxin. This suggests that oocytes could be an acceptable system in which to induce expression of binding sites.

Characteristics of toxin binding to rat brain and spinal cord membranes. Botulinum neurotoxin type B binding to rat membrane preparations increased as a function of protein. An apparent plateau was observed in the range of 20 to 30 μg membrane protein per assay (reaction volume, 100 μl). This result was obtained both with brain and with spinal cord. All subsequent experiments were done at a membrane protein concentration of 15 μg per assay.

The binding of botulinum neurotoxin to all membrane preparations increased with time, and a true equilibrium was not reached until 60 to 90 min. This incubation time was incorporated into subsequent experiments.

Temperature had a significant effect on specific binding. The greatest amount of specific binding was obtained at 23°C. This binding was reduced at 37°C, and it was markedly reduced at 0°C. All subsequent experiments were done at room temperature (~23°C).

Specific binding of the single chain and dichain species of botulinum neurotoxin type B were compared, using the assay conditions described in the preceding paragraphs. Regardless of the origin of tissues, the two species were indistinguishable in their binding characteristics. All subsequent experiments were done with the single chain molecule.

Various concentrations of iodinated botulinum neurotoxin type B were incubated with rat brain membrane preparations, and the resulting data on specific binding were used to generate a saturation isotherm and a scatchard plot. Graphic analysis of the data revealed a single class of binding sites with a Kd of 2 nM and a Bmax of 2.6 pmol/mg protein. In companion experiments, various concentrations of unlabeled toxin were used as competitive antagonists of labeled toxin binding. The apparent IC_{50} as deduced by graphic analysis was 2 nM, which is in accord with the Kd value for radioligand binding (see above).

Unlike homologous toxin, heterologous unlabeled toxin was not an effective antagonist of iodinated botulinum neurotoxin type B binding. At concentrations 10-fold greater than the IC_{50} for unlabeled serotype B, unlabeled serotypes A and C produced no more than 20 percent inhibition of iodinated toxin binding.

In addition to studies on membrane preparations derived from rat whole brain, work was done on preparations from select regions, including the forebrain, basal forebrain, cerebellum, and spinal cord (Table 1). The Kd values for these four regions ranged from 2.9×10^{-9} M (spinal cord) to 5.5×10^{-9} M (forebrain); the Bmax values ranged from 3.0 pmol/mg (forebrain) to 10.3 pmol/mg (cerebellum).

Botulinum toxin binding to mouse membrane preparations. A more limited series of studies was done on membrane preparations from different regions of mouse brain. Representative data for one specific region (saturation isotherm and resulting scatchard analysis) showed that mouse basal forebrain had a single class of high affinity binding sites for iodinated botulinum neurotoxin. The Kd value was 4.1 nM and the Bmax value was 8.4 pmol/mg protein, which are reasonably similar to the values for whole rat brain preparations.

When three specific tissues were analyzed (cerebellum, forebrain, basal brain), the resulting Kd values fell within a narrow range (2.9×10^{-9} M to 4.3×10^{-9} M), and this range fell within that observed for rat tissues (Table 1). On the other hand, the resulting Bmax values were more disparate. The difference between the mouse cerebellum (12 pmol/mg

protein) and the mouse forebrain (2 pmol/mg protein) was six-fold. The relatively high value obtained in mouse cerebellar membranes was exploited in later expression experiments (see below).

The effects of synaptotagmin antibodies on botulinum toxin binding and activity. Rat brain membrane preparations were incubated (30 min; 23°C) with antibody against either the cytosolic or lumenal domains of synaptotagmin. The antibodies were used at a five-fold excess to that necessary to detect antigen on Western blots (see below). Iodinated botulinum neurotoxin type B was then added (1×10^{-9} M) and binding was measured as described above. The results indicated that neither antibody reduced the amount of toxin binding.

In related experiments, phrenic nerve-hemidiaphragm preparations were immersed in medium at 5°C, which virtually abolishes both exocytosis and endocytosis. Antibody against either the cytosolic or lumenal domain of synaptotagmin was added, and incubation was continued for 30 min. Botulinum neurotoxin type B (1×10^{-11} M) was then added, and incubation was continued for an additional 30 min. The tissues were washed and suspended in antibody-free and toxin-free solution at 35°C, phrenic nerves were stimulated, and paralysis times were monitored. The results were: control tissues (n=10), 111±9 min; tissues (n=4) incubated with antibody against the cytosolic domain of synaptotagmin (118±12 min); tissues (n=6) incubated with antibody against the lumenal domain of the peptide (121±13 min). Neither antibody produced statistically significant protection against botulinum neurotoxin.

Botulinum toxin binding to human membrane preparations. Six different regions of human brain were randomly selected for study: prefrontal cortex, anterior temporal cortex, superior parietal cortex, putamen, globus pallidus, and cerebellum. Of these, the cerebellum was chosen as a prototype tissue on which to perform experiments that would define reaction conditions.

Botulinum neurotoxin type B binding to human cerebellar preparations increased as a

function of membrane protein concentration and time. An apparent plateau in binding was obtained at a protein concentration of 200 to 300 μg/assay. Subsequent experiments were done at a concentration of 100 μg/assay (100 μl). An apparent equilibrium was obtained between 90 and 120 min, and this incubation time was used in later experiments. To ensure that results with human tissues could be compared with those from laboratory animal tissues, experiments were done at room temperature (~23°C).

Saturation isotherm experiments were done under the conditions described above, and scatchard analyses were performed to characterize toxin binding to human cerebellum, frontal cortex and globus pallidus (Table 2). Interestingly, the average Kd value for the three regions of human brain (18.2 $\pm$ 5.0 nM) was significantly higher ($p<0.01$) than the average for the different regions of rat brain (4.2 $\pm$ 0.7 nM) and mouse brain (3.7 $\pm$ 0.9 nM). However, the Bmax values were reasonably similar to those seen with rodent tissues.

An effort was made to determine whether the amount of botulinum neurotoxin binding in brains of patients with no known neurologic disease was different from that in age- and sex-matched patients diagnosed with Alzheimer's disease. Because of the limited amount of tissue available from diseased patients, it was not possible to construct full binding curves for each brain region. Instead, a single toxin concentration was chosen for study (1×10^{-9} M), and the amount of specific binding (fmol/mg protein) was compared in six regions of control brain and Alzheimer disease brain. As shown in Table 3, there was a general pattern of decreased binding in Alzheimer's disease specimens. The decreases in the cerebellum ($p<0.005$), putamen ($p<0.025$), and especially the globus pallidus ($p<0.001$) were significant. In the latter case, binding was decreased by approximately an order of magnitude.

Because it was not possible to construct full binding curves for each region of brain from Alzheimer's disease patients, the underlying basis for decreased binding could not be established. However, preliminary experiments on cerebellum and putamen suggested that the major change was in Bmax rather than Kd. Additional work will be necessary to confirm this preliminary finding on all affected brain regions.

Expression of binding sites in oocytes. The binding of botulinum neurotoxin to Xenopus oocytes was examined before or after injection of mRNA (see Methods). A single toxin concentration was used (1×10^{-9} M), and conditions for the binding assay were identical to those used with rat brain tissues (see above).

As described earlier, membranes from native oocytes were devoid of specific binding sites. However, in most cases (>80%), injection of message from whole rat brain led to expression of specific binding sites. The average value obtained for membranes in 9 successful experiments was 30 ± 4 fmol/mg protein.

Earlier experiments on rat, mouse and human brain all demonstrated that cerebellum had the highest density of toxin binding sites. Therefore, mRNA from rat cerebellum or mouse cerebellum was injected into oocytes. Interestingly, the success rate in obtaining binding sites rose to 100% when cerebellar mRNA was injected. The actual values obtained were as follows: rat cerebellum mRNA, 40 ± 5 fmol/mg protein (n=4); mouse cerebellum mRNA, 42 ± 10 fmol/mg protein (n=4).

Synaptotagmin was either not expressed or only minimally expressed in native oocytes. However, injection of rat cerebellar mRNA into oocytes led to clear expression of the peptide. Both the cytosolic domain and the lumenal domain could be detected.

Binding experiments with botulinum neurotoxin type B were redone with oocytes injected with rat cerebellar mRNA. The amount of toxin binding was compared in control preparations, experimental preparations preincubated with antibody against the cytosolic domain of synaptotagmin, and experimental preparations preincubated with antibody against the lumenal domain of the peptide. Preincubation was for 30 min at 23°C; antibodies were used at a five-fold excess to that necessary to detect antigen in Western blots.

The amount of specific binding in the three tissue preparations was the same. There was no evidence for antagonism of toxin binding by either antibody.

DISCUSSION

Botulinum neurotoxin acts preferentially on cholinergic nerve endings to block acetylcholine release. The toxin produces this effect by proceeding through a sequence of events, the first of which is association with cell surface receptors. The identity of these receptors and their role in nerve function have not been determined (Middlebrook, 1989). Indeed, it has not even been established whether there are simple receptors. Montecucco (1986) has advanced the interesting idea that binding might be a biphasic process. There could be an initial interaction with lower affinity binding sites (viz., membrane lipids), and this would promote a secondary interaction with higher affinity sites (viz., protein). The value of this proposal and others will not be known until toxin receptors are actually identified.

A strategy for characterizing receptors. A number of investigators have studied labeled botulinum toxin binding to nerve membrane preparations (for representative studies, see Kozaki, 1979; Williams *et al.*, 1983; Evans *et al.*, 1986; Yokosawa *et al.*, 1991). Because the amount of nerve ending tissue at the neuromuscular junction is limited, almost the entire literature on binding deals with brain membrane preparations (but see the work by Black and Dolly, 1986a; Black and Dolly, 1986b). This is in keeping with the fact that botulinum toxin blocks transmitter release from isolated brain synaptosomes.

Earlier studies on botulinum toxin type B binding have shown that whole brain preparations have specific, high affinity binding sites. Furthermore, these binding sites are unique or relatively unique to this serotype, because heterologous serotypes do not block binding. These earlier studies have established a framework in which the current work was done. Botulinum neurotoxin type B was labeled to high specific activity, and its binding was studied in several discrete areas of the rat and mouse central nervous system. In addition, the present study contains the first experiments in which botulinum toxin binding has been measured in human tissues. This was done with specimens obtained from several regions of

human brain, and a comparison was made between tissues from patients without known neurological disease and tissues from patients with Alzheimer's disease. Finally, the present study contains the first data on botulinum toxin binding to Xenopus oocytes, a widely used expression system. This work was done both on native oocytes and on oocytes that had been injected with rat and mouse brain mRNA.

An initial examination of iodinated type B toxin binding showed that the single chain and dichain species were virtually indistinguishable. This is in marked contrast to findings with other steps in the paralytic process. To begin with, the single chain species does not appear to have the correct conformation and/or flexibility needed for cellular internalization. In addition, the single chain species expresses little or no enzymatic activity, perhaps because of conformational restraints on the catalytic domain. These limitations explain why the single chain molecule is much less potent than the dichain molecule when tested on phrenic nerve-hemidiaphragm preparations.

The membrane preparations used in the present study did not have intact synaptosomes, and thus there should be little possibility that the process of internalization could be mistaken for binding. However, to minimize any difficulties, the bulk of the work was done with the single chain molecule. This also reduced the prospect that toxin~substrate interactions could complicate matters because, as indicated above, the single chain molecule has little enzymatic activity.

There is one further respect in which the present study is an outgrowth of earlier work. In the recent past, Nishiki et al. (1993; 1994) have published work suggesting that synaptotagmin, perhaps in association with an auxiliary molecule, may act as a receptor for serotype B. Synaptotagmin is found in membranes of synaptic vesicles. During the process of exocytosis, vesicles meld with the plasma membrane. This means that for a finite period of time the lumenal domain of the molecule is exposed to the cell surface. It is at this point that, hypothetically, synaptotagmin could serve as a whole or partial receptor for the toxin. Therefore, botulinum neurotoxin binding (nerve tissue, oocytes) and biological activity

(phrenic nerve-hemidiaphragm) were studied in the absence and presence of antibodies directed against synaptotagmin. This work was intended to help clarify the issues that must be resolved before synaptotagmin can be regarded as an authentic receptor.

Analysis of neurotoxin binding. All regions of rat, mouse and human central nervous system that were tested had specific binding sites for botulinum neurotoxin type B. In general, the Kd values for the different regions of mouse and rat brain were comparable, but the Kd values for regions of human brain were higher.

There are three points that should be made in relation to the binding data. First, botulinum neurotoxin type B is orders of magnitude more potent in blocking mouse than blocking rat neuromuscular transmission (Burgen *et al.*, 1949). To the extent that binding to brain membranes reflects binding to motor nerve membranes, the data suggest that resistance of the rat is not due to an absence of specific receptors (and see Evans *et al.*, 1986). This conclusion meshes well with another line of recent research. Botulinum neurotoxin type B blocks transmission by cleaving synaptobrevin (also known as VAMP, or vesicle-associated membrane protein) at Gln^{76}-Phe^{77}. Mouse synaptobrevin has this bond, but rat synaptobrevin has a substitution at this site that creates a Val^{76}-Phe^{77} bond (Patarnello *et al.*, 1993). In all likelihood, resistance of the rat to serotype B is due to an absence of a susceptible cleavage site in the substrate and not to absence of specific receptors.

Second, botulinum toxin type B has substantially less affinity for binding sites in human brain than for sites in rat brain or mouse brain. The reason for this difference is not clear, but at least one possible explanation can be ruled out. One might argue that there are degradative changes in autopsy specimens, such as proteolysis of membranes, and thus high Kd values could be misleading. However, there is experimental evidence that weighs against this idea. Ligand binding studies with serotype A have shown that rat, mouse, and human brain membranes display nearly equivalent Kd values, and studies with serotype C have shown that human brain membranes actually display a higher affinity than rat or mouse brain (Coffield *et*

al., submitted for publication). These results are not consistent with a hypothesis of generalized membrane deterioration in human specimens.

Third, the data do not support the idea that synaptotagmin acts as a sole receptor for botulinum neurotoxin type B. Experiments in which antibodies against the lumenal and cytosolic domains of synaptotagmin were tested as antagonists of toxin binding to brain membrane preparations did not reveal any protection. The antibodies--like the toxin--are large molecules (>150,000 daltons), which means that antibodies could block toxin binding either by attaching to the toxin binding site (viz., true competition) or by attaching to another site (viz., steric hindrance). Therefore, a complete absence of protection raises questions about whether the peptide is acting as a sole receptor.

The binding studies on brain membrane preparations were supplemented by experiments on oocytes and on phrenic nerve-hemidiaphragm preparations. Native oocytes did not express specific receptors for toxin, but injection of mouse or rat cerebellar mRNA did lead to expression of binding sites. In keeping with the results on brain membranes, antibodies against the two free domains of synaptotagmin did not diminish toxin binding to oocyte membranes. Analogous results were obtained in functional studies with a neuromuscular preparation. Antibodies against synaptotagmin did not alter the amount of time necessary for toxin-induced blockade of transmission in the phrenic nerve-hemidiaphragm.

The neuromuscular junction studies are especially relevant to issues that pertain to toxin binding. As discussed above, it is presumably the lumenal domain of synaptotagmin that would serve as a binding site, and this portion of the molecule would be exposed when exocytotic vesicles meld with the plasma membrane. Furthermore, one would predict that the number of these binding sites would be relatively low under conditions in which there is little or no exocytosis (e.g., low temperature), but the relative number and rate of turnover would increase when there is substantial exocytosis (e.g., rapid nerve stimulation). Indeed, recent work has demonstrated exactly this outcome. De Camilli and his colleagues (Mundigl *et al.*, 1995) have shown that an iodinated antibody against the lumenal domain of synaptotagmin can

be used to measure exocytosis. This work has shown that binding is reduced at low temperature, but binding of the antibody increases at physiological temperature or with nerve depolarization.

Experiments in the present study involved the same antibody as that developed to measure exocytotic activity. The antibody was added to the neuromuscular junction at 4°C for 30 min, which is more than adequate time for equilibrium to be obtained (ca. 3 to 4 min; Mundigl *et al.*, 1995). Botulinum toxin was then added for 30 min, after which tissues were washed and suspended in physiological solution at 35°C. The results demonstrated that prior treatment of tissues with antibodies against synaptotagmin did not afford any protection against botulinum toxin type B.

The combined results raise serious questions about the role of synaptotagmin as a sole receptor for botulinum toxin. However, they do not necessarily challenge the premise that the peptide could be one part of a dual receptor. The absence of antibody protection in binding experiments could be explained on the basis that toxin was associating with the non-synaptotagmin portion of the dual receptor. The absence of protection in functional experiments could be explained on the same basis, with the assumption that the toxin becomes associated with newly exposed synaptotagmin when nerve stimulation begins.

One possible approach to determining whether synaptotagmin is part a dual receptor would be to utilize the techniques of molecular biology. If a tissue that is sensitive to type B toxin could be rendered free of synaptotagmin (e.g., deletion mutant or antisense treatment), this would allow one to determine whether the peptide plays an essential role in toxin binding. Efforts are currently underway to generate such a preparation.

Toxin binding and neurologic disorders. An analysis of toxin binding in brain specimens from patients with no known neurological disease and in specimens from patients with Alzheimer's disease revealed profound changes. It was generally true that binding was decreased in Alzheimer's disease, but because of variability in the data some of the differences

did not attain statistical significance. This was not the case for cerebellum and putamen, where the differences were significantly different ($p < 0.005$ and 0.025, respectively), or for the globus pallidus, where the difference was striking ($p < 0.001$).

Given the nature of the disease under study, one might speculate that these changes are merely a reflection of cell death. Cell loss is a characteristic feature of Alzheimer's disease, and thus loss of toxin binding sites might be a predictable and mundane observation. However, there is a compelling reason for not accepting this line of speculation. The substantial neurohistological literature on Alzheimer's disease shows that higher centers, such as cerebral cortex, are particularly vulnerable to cell loss. There is no evidence that areas such as the globus pallidus are preferentially affected while areas such as the cortex are spared. The toxin binding data appear to be the reverse of what one would predict if the results were merely a reflection of the characteristic pattern of cell death in Alzheimer's disease.

There is not enough information to explain the provocative data on toxin binding. Instead, there is a need to broaden the scope of investigation. To begin with, the original work should be reproduced on Alzheimer's patients from other geographic areas to determine whether the present patients and findings are generalizable. In addition, the approach should be reproduced with other toxin serotypes to determine whether the results with serotype B are unique. If the finding of decreased toxin binding in non-cortical areas proves to be reproducible, then the problem should be pursued vigorously. There is the possibility that botulinum toxin binding may be a biomarker for some aspect of Alzheimer's disease.

Expression of binding sites. Native Xenopus oocytes were devoid of specific binding sites for botulinum neurotoxin type B. This, along with findings on frog brain and spinal cord, show that toxin association with membranes is not a universal phenomenon. Thus far, specific association has been observed only in situations in which the toxin blocks exocytosis (see above).

Injection of oocytes with mRNA of whole rat brain origin led to expression of binding

sites, and injection of mRNA of either rat cerebellum or mouse cerebellum origin led to expression of even more binding sites. These results are encouraging, not only because they demonstrate that binding sites can be induced in oocytes, but also because they demonstrate that the relative density of binding sites in the expression system mimics the relative density in the donor tissue.

The fact that oocytes express toxin receptors has many implications, the most obvious being that the expression system can be used to test hypotheses about the identity of toxin receptors. Recent work by Nishiki *et al.* (1993; 1994) has led to the proposal that synaptotagmin, perhaps in association with an auxiliary molecule, may be a toxin receptor. If this were true, one would expect that injection and expression of the gene for synaptotagmin and the auxiliary molecule should lead to appearance of binding sites. Alternatively, injection of total mRNA simultaneously with an antisense message to synaptotagmin should block expression of binding sites. Experiments along these lines are in progress, but another line of work may also be revealing. As reported above, the frog lacks specific binding sites for serotype B, and the frog neuromuscular junction is resistant to the toxin. Nevertheless, the frog neuromuscular junction reacts positively in immunohistochemistry stains with anti-synaptotagmin antibody (Coffield, Bakry, Zhang and Simpson, manuscript in preparation). This suggests that synaptotagmin by itself cannot be a fully competent toxin receptor.

ACKNOWLEDGMENT

The authors are grateful to Dr. Andrew B. Maksymowych for assistance in performing electrophoresis.

REFERENCES

BAKRY, N., KAMATA, Y. AND SIMPSON, L. L. Lectins from *Triticum vulgaris* and *Limax flavus* are universal antagonists of botulinum neurotoxin and tetanus toxin. J. Pharmacol. Exp. Ther. **258**: 830-836, 1991a.

BAKRY, N., KAMATA, Y., SORENSEN, R. AND SIMPSON, L. L. Tetanus toxin and neuronal membranes: the relationship between binding and toxicity. J. Pharmacol. Exp. Ther. **258**: 613-619, 1991b.

BINZ, T., BLASI, J., YAMASAKI, S., BAUMEISTER, A., LINK, E., SUDHOF, T. C., JAHN, R. AND NIEMANN, H. Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J. Biol. Chem. **269**: 1617-1620, 1994.

BLACK J. D. AND DOLLY, J. O. Interaction of -I-labeled botulinum neurotoxins with nerve terminals. I. Ultrastructural autoradiographic localization and quantitation of distinct membrane acceptors for types A and B on motor nerves. J. Cell Biol. **103**: 521-534, 1986a.

BLACK J. D. AND DOLLY, J. O. Interaction of -I-labeled botulinum neurotoxins with nerve terminals. II. Autoradiographic evidence for its uptake into motor nerves by acceptor-mediated endocytosis. J. Cell Biol. **103**: 535-544, 1986b.

BLASI, J., CHAPMAN, E. R., LINK, E., BINZ, T., YAMASAKI, S., DE CAMILLI, P., SüDHOF, T. C., NIEMANN, H. AND JAHN, R. Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature **365**: 160-163, 1993a.

BLASI, J., CHAPMAN, E. R., YAMASAKI, S., BINZ, T., NIEMANN, H. AND JAHN, R. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO Journal **12**: 4821-4828, 1993b.

BURGEN, A. S. V., DICKENS, F. AND ZATMAN, L. J. The action of botulinum toxin on the neuromuscular junction. J. Physiol. **109**: 10-24, 1949.

COFFIELD, J. A., CONSIDINE, R. V., JEYAPAUL, J. AND SIMPSON, L. L. The role of transglutaminase in the mechanism of action of botulinum neurotoxin and tetanus toxin. J. Biol. Chem. **269**: 24454-24458, 1994.

DASGUPTA, B. R. The structure of botulinum neurotoxin. In Botulinum Neurotoxin and Tetanus Toxin, ed. by L. L. Simpson, pp. 53-67, Academic Press, San Diego, 1989.

EVANS, D. M., WILLIAMS, R. S., SHONE, C. C., HAMBLETON, P., MELLING, J. AND DOLLY, J. O. Botulinum neurotoxin type B. Its purification, radioiodination and interaction with rat-brain synaptosomal membranes. Europ. J. Biochem. **154**: 409-416, 1986.

HABERMANN, E. AND DREYER, F. Clostridial neurotoxins: Handling and action at the cellular level. Curr. Topics. Microbiol. Immunol. **129**: 93-179, 1986.

KOZAKI, S. Interaction of botulinum type A, B and E derivative toxins with synaptosomes of rat brain. Naun-Schmiedebergs Archiv. Pharmacol. **308**: 67-70, 1979.

MCPHERSON, G. A. EBDA, User Manual Computer Program, 1982.

MIDDLEBROOK, J. L. Cell surface receptors for protein toxins. In Botulinum Neurotoxin and Tetanus Toxin. ed. by L. L. Simpson, pp. 95-119, Academic Press, San Diego, 1989.

MONTECUCCO, C. How do tetanus and botulinum toxins bind to neuronal membranes? Trends Biochem. Sci. **11**: 314-317, 1986.

MONTECUCCO, C. and SCHIAVO, G. Mechanism of action of tetanus and botulinum neurotoxins. Molecular Microbiology **13**: 1-8, 1994.

MUNDIGL, O., VERDERIO, C., KRASZEWSKI, K., DE CAMILLI, P., AND MATTEOLI, M. A radioimmunoassay to monitor synaptic activity in hippocampal neurons *in vitro*. E. J. Cell Biol. **66**: 246-256, 1995.

NISHIKI, T., OGASAWARA, J., KAMATA, Y., AND KOZAKI, S. Solubilization and characterization of the acceptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptic membranes. Biochem. Biophys. Acta **1158**: 333-338, 1993.

NISHIKI, T., KAMATA, Y., NEMOTO, Y., OMORI, A., ITO, T., TAKAHASHI, M. AND KOZAKI, S. Identification of protein receptor for *Clostridium botulinum* type B neurotoxin in rat brain synaptosomes. J. Biol. Chem. **269**: 10498-10503, 1994.

PATARNELLO, T., BARGELLONI, L., ROSSETTO, O., SCHIAVO, G., AND MONTECUCCO, C. Neurotransmission and secretion. Nature **364**: 581-582, 1993.

SAKAGUCHI, G. *Clostridium botulinum* toxins. Pharmacol. Therapeut. **19**: 165-194, 1982.

SCHIAVO, G., BENFENATI, F., POULAIN, B., ROSSETTO, O., POLVERINO DE LAURETO, P., DASGUPTA, B. R. AND MONTECUCCO, C. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature **359**: 832-835, 1992.

SCHIAVO, G., ROSSETTO, O., CATSICAS, S., DE LAURETO, P. P., DASGUPTA, B. R., BENFENATI, F. AND MONTECUCCO, C. Identification of the nerve terminal targets of botulinum neurotoxin serotypes A, D and E. J. Biol. Chem. **268**: 23784-23787, 1993a.

SCHIAVO, G., SHONE, C. C., ROSSETTO, O., ALEXANDER, F. C. G. AND MONTECUCCO, C. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. J. Biol. Chem. **268**: 11516-11519, 1993b.

SCHIAVO, G., MALIZIO, C., TRIMBLE, W. S., POLVERINO DE LAURETO, P., SUGIYAMA, H., JOHNSON, E. A. AND MONTECUCCO, C. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. J. Biol. Chem. **269**: 20213-20216, 1994.

SIMPSON, L. L. Botulinum Neurotoxin and Tetanus Toxin, pp. 1-422, Academic Press, San Diego, 1989.

SIMPSON, L. L. Current concepts on the mechanism of action of clostridial neurotoxins. In Botulinum and Tetanus Neurotoxins: Neurotransmission and Biomedical Aspects, ed. by B. R. DasGupta, pp. 5-15, Plenum Publishing Corporation, New York, 1993.

SIMPSON, L. L. AND DASGUPTA, B. R. Botulinum neurotoxin type E: studies on mechanism of action and on structure-activity relationships. J. Pharmacol. Exp. Ther. **224**: 135-140, 1983.

SIMPSON, L. L., SCHMIDT, J. J. AND MIDDLEBROOK, J. L. Isolation and characterization of the botulinum neurotoxins. Methods Enzymol. **165**: 76-85, 1988.

WILLIAMS, R. S., TSE, C. K., DOLLY, J. O., HAMBLETON, P. AND MELLING, J. Radioiodination of botulinum neurotoxin type A with retention of biological activity and its binding to brain synaptosomes. Europ. J. Biochem. **131**: 437-445, 1983.

YAMASAKI, S., BAUMEISTER, A., BINZ, T., BLASI, J., LINK, E., CORNILLE, F., ROQUES, B., FYKSE, E. M., SüDHOF, T. C., JAHN, R. AND NIEMANN, H. Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. J. Biol. Chem. **269**: 12764-12772, 1994a.

YAMASAKI, S., BINZ, T., HAYASHI, T., SZABO, E., YAMASAKI, N., EKLUND, M., JAHN, R. AND NIEMANN, H. Botulinum neurotoxin type G proteolyses the Ala81-Ala82 bond of rat synaptobrevin 2. Biochem. and Biophys. Res. Comm. **220**: 829-835, 1994b.

YOKOSAWA, N., TSUZUKI, K., SYUTO, B., FUJII, N., KIMURA, K. AND OGUMA, K. Binding of botulinum type Cl, D and E neurotoxins to neuronal cell lines and synaptosomes. Toxicon **29**: 261-264, 1991.

TABLE 1

Specific binding of ^{125}I-botulinum neurotoxin type B to different areas of rat and mouse central nervous system

Tissue	Kd (neuromuscular)[a]	Bmax (pmol/mg protein)[a]
Rat forebrain	5.5 ± 1.0	3.0 ± 0.3
Rat basal forebrain	4.8 ± 1.1	7.7 ± 0.8
Rat cerebellum	3.5 ± 0.4	10.3 ± 0.6
Rat spinal cord	2.9 ± 0.3	8.0 ± 1.0
Average	4.2 ± 0.7	7.3 ± 0.7
Mouse forebrain	2.9 ± 0.9	2.0 ± 0.6
Mouse basal forebrain	4.1 ± 1.2	8.4 ± 1.5
Mouse cerebellum	4.3 ± 0.6	12.0 ± 3.5
Average	3.7 ± 0.9	7.5 ± 1.9

[a] Values are expressed as the mean ± SEM. N = 3 or more per group.

TABLE 2

Specific binding of ^{125}I-botulinum neurotoxin type B to different areas of human brain

Tissue	Kd (neuromuscular)[a]	Bmax (pmol/mg protein)[a]
Frontal cortex	16.6 ± 7.0	2.5 ± 1.4
Globus pallidus	24.0 ± 4.0	2.0 ± 0.5
Cerebellum	14.0 ± 4.0	5.2 ± 2.0
Average	18.2 ± 5.0	3.2 ± 1.3

[a] Values are expressed as the mean ± SEM. N = 3 or more per group.

TABLE 3

Specific binding of ^{125}I-botulinum neurotoxin type B to tissues from Control Patients and Alzheimer's Disease Patients

Tissue	Control[a]	Alzheimer's Disease[a]
Prefrontal Cortex	57 ± 2.8 (10)	50 ± 1.9 (9)
Anterior Temporal Cortex	57 ± 2.6 (6)	39 ± 6.7 (4)
Superior Parietal Cortex	35 ± 2.0 (6)	28 ± 2.0 (4)
Cerebellum	72 ± 1.8 (15)	40 ± 1.1 (10)
Putamen	40 ± 1.2 (6)	23 ± 5.3 (4)
Globus Pallidus	50 ± 2.7 (7)	5 ± 1.4 (7)

[a] Values (mean ± SEM) are expressed as fmol/mg protein. Numbers in parentheses represent group N.